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Liposome Targeting to Tumors using Vitamin and Growth Factor Receptors

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Liposome-encapsulated anticancer drugs reveal their potential for increased therapeutic efficacy and decreased nonspecific toxicities due to their ability to enhance the delivery of chemotherapeutic agents to solid tumors. Advances in liposome technology have resulted in the development of ligand-targeted liposomes capable of selectively increasing the efficacy of carried agents against receptor-bearing tumor cells. Receptors for vitamins and growth factors have become attractive targets for ligand-directed liposomal therapies due to their high expression levels on various forms of cancer and

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their ability to internalize after binding to the liposomes conjugated to receptors' natural ligands (vitamins) or synthetic agonists (receptor-specific antibodies and synthetic peptides). This chapter summarizes various strategies and advances in targeting liposomes to vitamin and growth factor receptors *in vitro* and *in vivo* with special emphasis on two extensively studied liposome-targeting systems utilizing folate receptor and HER2/neu growth factor receptor.

I. Introduction

Growth, nutrition, and differentiation are among the key functions of living cells that constitute our body. Energy-rich nutrients and structural building blocks, such as sugars, fats, and amino acids, are needed in abundance and enter cells from a lavish extracellular pool. To effectively metabolize the nutrients and build its own bulk, the cell needs vitamins and other enzyme cofactors that are not produced by the cell itself and must be absorbed from a relatively scant environment. Raw power of cellular growth provided by metabolism of nutrients is tamed by the process of cell growth control and differentiation based on intricate communication between the often remote groups of cells through hormones, chemical effectors, and growth factors. Cellular uptake of vitamins and response to hormones and effectors depends on receptor proteins that specifically interact with these substances and elicit proper physiological responses on the cellular level.

Growth and differentiation of the cells are the first functions to change dramatically when the cells become malignant. In malignant cells, the molecular machinery of hormone and growth factor receptors is changed to provide constant stimulation of unabridged cell growth and reduced ability for normal differentiation. Intensive biosynthesis of cellular components, in turn, requires increased supply of metabolic cofactors. Thus, normal and malignant cells often have profound differences in the abundance and function of vitamin and growth factor receptors. Because of these differences and because the receptors for water-soluble, hydrophilic molecules such as peptide hormones and vitamins are usually exposed at the cell surface, vitamin and growth factor receptors are promising "recognition tags" for targeted anticancer drug delivery.

In the malignant phenotype the expression levels of certain vitamin and growth factor receptors are often substantially elevated (Sporn and Roberts, 1985; Slamon *et al.*, 1987, 1989; Berchuck *et al.*, 1990; Weitman *et al.*, 1992; Ross *et al.*, 1994; Fan and Mendelsohn, 1998).

Antibodies, antibody fragments, and small molecule ligands with affinities to cell-surface receptors for vitamins and growth factors have been used to target a variety of toxins (Leamon and Low, 1992; Leamon *et al.*, 1993; Ramakrishnan *et al.*, 1996; Rosenblum *et al.*, 1999), radionuclides (Hartman *et al.*, 1994; Wilder *et al.*, 1996; Multani *et al.*, 1998; Iznaga-Escobar, 1998; Wilbur *et al.*, 1999), enzymes (Rodrigues *et al.*, 1995; Jinno *et al.*, 1996; Lu *et al.*, 1999) and small-molecule therapeutics (Sivam *et al.*, 1995; Uckun *et al.*, 1998; Tolcher *et al.*, 1999) specifically to receptor-overexpressing cancer cells. There are some limitations inherent to each of these approaches Toxin conjugates, for example, age quite immunogenically and even at subtherapeutic doses still have nonspecific toxicity characteristic for the toxin domain. Frequent toxicity in this case is vascular leak syndrome (VLS), characterized by hypoalbuminemia, weight gain, hypotension, and peripheral and pulmonary edema resulting from extravasation of fluid and proteins from the vascular compartment (Vitetta *et al.*, 1991; Grossbard *et al.*, 1992, 1993; Sausville *et al.*, 1995; Stone *et al.*, 1996). Cancer cell-specific radionuclide–antibody conjugates have shown significant promise in the treatment of hematological cancers but are associated with significant hematological toxicities, are limited by the so-called binding-site barrier in solid tumors (Weinstein *et al.*, 1987), and may be limited to use in large clinical centers due to the need for complex dosimetry calculations and an on-site radiopharmacy (Multani and Grossbard, 1998; Iznaga-Escobar, 1998). All these types of targeted drug-delivery systems are limited by their rapid clearance from the circulation, low number of active drug delivered per targeting event (interaction of the targeted drug carrier with the target cell), and potential inactivation of the cytotoxic agent upon coupling to a targeting ligand or upon exposure to the biological media after administration into the body. These limitations, however, are not inherent to drug-delivery systems based on liposomes.

Liposomes were first described in 1960s when Bangham and co-workers discovered that lecithin swelling in aqueous buffers forms microscopic bodies composed of nested lipid bilayers enclosing aqueous interior (Bangham, 1963; Bangham *et al.*, 1965). During more than 3 decades since this discovery, liposomes have been the subject of numerous studies, books, and reviews. For details of current liposome technology we refer the reader to an excellent and comprehensive textbook by Lasic (1993) and to a recent book that offers state-of-the-art coverage of liposome drug delivery in general (Lasic and Papahadjopoulos, 1998).

Generally, liposomes are vesicular structures consisting of one or more enclosed lipid-bilayer membranes (lamellae) that encircle an

aqueous space containing the solute of interest, which in the context of this chapter is an anticancer active principle, whether it is a small-molecular-weight drug or a large macromolecule line DNA. Liposomes are generally produced when certain lipids, especially the natural lipid components of biomembranes, e.g., phosphatidylcholine, are allowed to swell in aqueous buffers and then are fragmented by mechanical shearing, ultrasonication, microfluidization, or extrusion through micro- or nanoporous membranes; alternatively, the lipids are solubilized in the presence of a dialyzable detergent which is then removed to allow the lipid molecules to associate in bilayers and form vesicles. Lipids in the membranes of liposomes are organized in two-dimensionally oriented (liquid crystalline) phases not unlike biological membranes; this property of liposomes prompted their use as a model of biomembranes in many biophysical and biochemical studies (Lasic, 1993).

The microcontainer nature of liposomes and their natural compatibility with living tissues makes them ideally suited as drug carriers. Water-soluble drugs can be loaded into liposomes simply by sequestering (entrapment) drug solution within liposomes during their formation. Moderately lipophilic drugs having the properties of weak acids or bases can be very effectively encapsulated by "active" or "remote" loading methods by creating a transmembrane gradient of pH and/or electrochemical potential that "drives" the drug into the liposome (Nichols and Deamer, 1976; Mayer *et al.*, 1985; Haran *et al.*, 1993). More lipophilic drugs with poor water solubility usually associate with the liposome bilayer.

Despite common structural features such as vesicular structure and bilayer organization of lipids, various types of liposomes differ with respect to size (from 30 nm to several micrometers), number of bilayers (unilamellar, oligolamellar, or multilamellar), lipid composition, surface charge, and presence or absence of so-called steric stabilization by the surface-attached sugar or hydrophilic polymers which may substantially improve the fate of drug-carrying liposomes in the body (Fig. 1). Common lipid components of liposome membranes, as mentioned above, are phospholipids, such as phosphatidylcholine, and sterols, such as cholesterol. Cholesterol is an important component of liposomal formulations because it reduces membrane permeability (Papahadjopoulos *et al.*, 1972; Mayhew *et al.*, 1979), and thus increases stability of encapsulated drug in the circulation, and because it limits the exchange of membrane components out of the liposomal membrane in blood plasma (Allen, 1981; Damen *et al.*, 1981). Anticancer drugs are commonly formulated into unilamellar liposomes 70–150 nm in size which allows them to permeate through the vasculature of tumors but

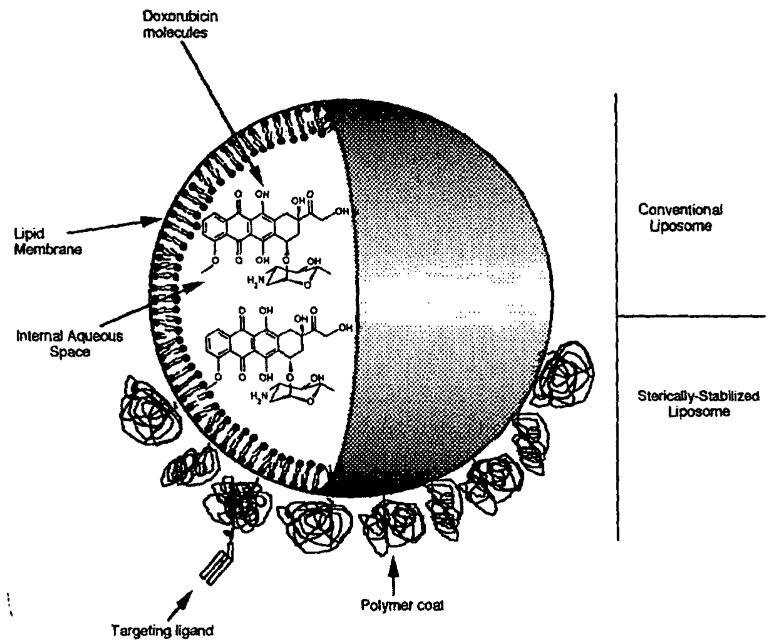

FIG. 1. Schematic representation of a drug-loaded liposome both with poly(ethylene glycol) coating (sterically stabilized liposome, SSL) or without coating ("conventional" liposome). The liposome has a lipid bilayer membrane that encapsulates internal aqueous space used to hold drug substances. Some amphiphilic drugs, such as doxorubicin, can be encapsulated at concentrations exceeding their aqueous solubility and form crystals in the liposome interior. Yet more hydrophobic drugs may be carried within the lipid bilayer. Further modification of the surface by covalently attaching targeting ligands such as small-molecule ligands (e.g., folic aid) or antibody fragments can result in liposomes that are specifically endocytosed by target cells that express a receptor for that ligand or an antigen for the antibody, i.e., folate receptor or growth factor receptor. Phospholipids, such as distearoylphosphatidylcholine (DSPC) and other phosphatidylcholines (lecithins), as well as cholesterol, are common components of systemically delivered liposome formulations, although other lipid compositions are possible.

not of normal tissues (Hobbs *et al.*, 1997). These liposomes are administered intravenously and should be able to persist in the circulation long enough to allow liposome extravasation into the tumor. Early liposomal formulations suffered from fast clearance from circulation by the cells of mononuclear phagocytic system. Significant advance in liposome technology was the discovery that coating of liposome surface with some oligosaccharides or polymers, especially poly(ethylene glycol)

("sterically stabilized liposomes") results in remarkably low blood-clearance rates (Gabizon and Papahadjopoulos, 1988; Klibanov *et al.*, 1990; Allen *et al.*, 1991; Papahadjopoulos *et al.*, 1991, Woodle and Lasic, 1992). This discovery, along with the development of highly efficient methods of "active" loading of drugs into liposomes (Cullis *et al.*, 1997) aided in the development of liposomal drugs all the way to clinic.

Clinical trials with liposomal drugs such as doxorubicin and vincristine have shown either similar or increased therapeutic efficacy when compared to the free drug, while significantly altering the toxicity profile and reducing many of the common nonspecific toxicities associated with the free drug (Muggia *et al.*, 1997; Ranson *et al.*, 1997; Gabizon, 1998; Northfelt *et al.*, 1998; Drummond *et al.*, 1999; Gelmon *et al.*, 1999; Shapiro *et al.*, 1999; Valero *et al.*, 1999). These benefits result from a variety of factors, the major one being the substitution of tumor tissue-specific biodistribution of the drug-loaded liposomes for a relatively nonspecific biodistribution of the drug itself (Hwang, 1987; Allen and Papahadjopoulos, 1993; Allen *et al.*, 1995a). Such alteration of the drug biodistribution is possible because of the drug persistence within the circulating liposomes, higher vascular permeability of tumor vessels for microparticles, and low clearance rate of liposomes from tumors which lack lymphatic drainage (for recent reviews see Gabizon *et al.*, 1997; Allen, 1998; Bally *et al.*, 1998; Martin, 1998; Drummond *et al.*, 1999; Gabizon and Barenholz, 1999). The net effect is an increased and selective extravasation of drug-loaded liposomes at the tumor site and an increased accumulation of liposomal drugs at the tumor. This "passive" targeting of liposomes to solid tumors not only increases the therapeutic index of liposome-loaded anticancer drugs, but also enables and benefits further "active," ligand–directed targeting of drug-loaded liposomes to cancer cells.

Besides their propensity for extravasation into malignant rather than normal tissues, liposomes offer several other advantages as drug carriers for ligand-directed targeting (Rosenberg *et al.*, 1987; Lee and Low, 1994, 1995; Park *et al.*, 1995, 1997a, 1998a; Kirpotin *et al.*, 1997a, 1998). Liposomes can deliver much larger drug payloads per each targeting event. For example, one doxorubicin-loaded liposome internalized into cancer cells via a ligand–receptor interaction carries into the cell approximately 2×10^5 molecules of the drug. Liposomes can protect the encapsulated drug from degradation by enzymes and neutralization by antibodies in the central compartment until the drug enters the target cell.

Ligand-directed targeting may increase the bioavaliability of liposomal drug to target cells. For the drug to work, it must be released from the carrier upon reaching the target tissue. Following extravasation, nontargeted liposomes are primarily found in the tumor interstitium surrounding the cancer cells. From relatively stable doxorubicin formulations, such as Doxil (Alza Corp.) the drug is released slowly in the area of close proximity to the tumor, where the free drug may then diffuse into the nearby cancer cells. Liposomes may also be modified for targeting to specific ligands on various target cells by either covalent or noncovalent conjugation of a targeting ligand to the liposome surface. If the liposome is targeted via conjugated ligands to internalizing receptors located preferentially on cancer cells, breakdown of the liposome and release of the drug occurs intracellularly, effectively increasing the amount of bioavailable drug (Machy *et al.*, 1982; Lee *et al.*, 1998; Drummond *et al.*, 1999). For this approach to be effective, the drug must be stable in the acidic environment of the endosomes and lysosomes and in the presence of degradative enzymes located in these intracellular organelles. Luckily, current anticancer drugs of choice for liposomal delivery, such as doxorubicin, daunorubicin, and vinca alkaloids, can escape the organelles of endocytic pathway intact; other drugs, however, such as cytosine arabinoside, may be degraded, resulting in significantly diminished activity (Huang *et al.*, 1983). In addition, liposome internalization may simply serve to limit the diffusion of the released drug away from the tumor, thus exposing more of the tumor to the cytostatic agent (Allen *et al.*, 1998). Active targeting of liposomes will also be important in treating hematological cancers where blood-borne malignant cells will be unable to benefit from the passive targeting approach used in treating solid tumors. Allen and co-workers have recently demonstrated encouraging results targeting liposomes to internalizing CD19 protein exposed on malignant B-cells *in vivo* (Lopes de Menezes *et al.*, 1998).

The above considerations indicate that the efficacy of ligand-directed liposome targeting is higher if after binding to the surface of the target cell the liposome becomes internalized by this cell. Because vitamin receptors often perform cellular transport function and growth factor–receptors often perform cellular transport function and growth factor–receptor complexes are often internalized by the cell as part of cellular response it is more likely that liposomes conjugated to ligands specific to these receptors will be also internalized and will satisfy this requirement. A number of studies where liposomes or lipid complexes were targeted to vitamin and growth factor receptors on cancer cells is given in Table I. Folate receptor is the only vitamin receptor with reported use in liposome targeting, although the use of other vitamin receptors such as that for pyridoxal phosphate or pyridoxine have been mentioned (Zalipsky *et al.*, 1998). Growth factor receptors reported in liposome

TABLE I
LIGANDOLIPOSOMES TARGETED TO VITAMIN AND GROWTH FACTOR RECEPTORS

Receptor	Liposome composition	Substances delivered	Targeting ligand	Cell lines	Reference
FR	DSPC:Chol	Doxorubicin; marker	Folic acid	KB (human nasopharyngeal carcinoma), HeLa (human cervical carcinoma)	(Lee and Low, 1994, 1995); Wang and Low, (1998)
	HSPC:Chol:PEG-DSPE	Doxorubicin; marker	Folic acid	KB	Gabizon *et al.* (1999)
	Diplasmenyl(C_{16}) choline: dihydroChol	Ara-C; marker	Folic acid	KB	Rui *et al.* (1998)
	DSPC:Chol	Oligonucleotides	Folic acid	KB	Wang *et al.* (1995)
	Anionic liposome	plasmid DNA–polylysine complex	Folic acid	KB	Lee and Huang (1996)
HER-2/*neu*	PC:Chol; PC:Chol:PEG-DSPE (PC = POPC, HSPC, DSPC)	Doxorubicin; marker	Anti-HER2 Fab′ Anti-HER2 scFv	SKBR-3, MCF-7, MCF/7HER2; MBA-MD-453; BT-474 (human breast carcinomas); WI-38 (normal human lung)	Kirpotin *et al.* (1997a, 1997b, 1998, 2000); Park *et al.* (1995, 1997, 1998a, 1998b, 2000)
	HSPC:Chol:PEG-DSPE	Doxorubicin, marker	Anti-HER2 IgG	N-87 (human gastric carcinoma)	Goren *et al.* (1996)
	DOTAP:DOPE:PEG-DSPE	Phosphorothiate oligonicleotide	Anti-HER2 Fab′	SKBR-3	Meyer *et al.* (1998)
	DDAB:Chol; DDAB:Chol:PEG-DSPE	Plasmid DNA	Anti-HER2 Fab′	SKBR-3	Park *et al.* (1997)
EGFRr	DPPC:Chol	[^{3}H]-inulin (marker)	Human EGF	Fibroblasts	Ishii *et al.* (1989)
	DSPC:Chol:PEG-DSPE	^{125}I-labeled antibody	Anti-human EGFR IgG (C225)	DU-145 (human prostate carcinoma	Harding *et al.* (1997)
	DC-Chol:DOPE	Plasmid DNA	Human EGF	HEC-1A (adenocarcinoma) GCH-1 (human chorionic carcinoma)	Kikuchi *et al.* (1996)
VEGFR	DSPC:Chol	Anti-VEGF nucleic acid aptamers	Anti-VEGF nucleic acid aptamers	HUVEC (human vascular endothelium)	Willis *et al.* (1998)
NGFR	EggPC:DPPE:Chol	FITC-dextran	Mouse NGF	PC12 (pheochromocytoma), HS294 (human melanoma)	Rosenberg *et al.* (1987)

targeting studies include the epidermal growth factor receptor, vascular endothelial growth factor receptor, and nerve growth factor receptor (Table I). A variety of different ligands have been used to target these receptors, including antibodies or antibody fragments, small molecules such as folic acid, small peptides/proteins such as the native growth factors, and nucleic acid aptamers. The two most well studied of these approaches are the folate-targeted liposomes using the small-molecule folic acid tethered to the end of a PEG-linked lipid anchor and the anti-HER2 Fab′ or single-chain Fv-targeted liposomes (Table I). These two approaches make excellent case studies outlining general methodology in liposome targeting to internalizable cell surface epitopes using small effector molecules or an antibody and are discussed at length in the following sections in comparison to other modes of liposome targeting. It should be noted, however, that although the targeting of growth factor receptors has been primarily accomplished with antibodies or antibody fragments, and the targeting of vitamin receptors with their low-molecular-weight ligands, these approaches are not exclusive. For example, it is also possible and maybe even desirable in some instances to target growth factor receptors with their small-peptide ligands (for example, heregulin, EGF, NGF, and VEGF) or vitamin receptors with antibodies against appropriate receptors.

II. Targeting of Liposomes to Vitamin Receptors: The Case of Folic Acid

A. Folate Receptor as Internalizing Target on Malignant Cells

The vitamin folic acid and its reduced derivatives can be taken up by cells using both a low-affinity ($K_d \sim 1$–$5\,\mu M$) transmembrane protein responsible for the passive diffusion of reduced folates across the plasma membrane of cells and a high-affinity GPI-anchored protein ($K_d \sim 0.01$–$1\,nM$) that accumulates folic acid in the cell following receptor-mediated endocytosis by a clathrin-independent pathway (Kane *et al.*, 1986, 1989; Kamen *et al.*, 1988). The former is referred to as the reduced folate carrier (RFC) and demonstrates a considerable preference for reduced folates, such as 5-methyltetrahydrofolate, methotrexate, and 5-formyltetrahydrofolate, compared to free folic acid (Henderson, 1990; Antony, 1992). The RFC is unable to bind or mediate the uptake of FA conjugates. The folate-binding protein (FBP), also referred to as the folate receptor (FR), is actually a class of receptors expressed in low levels on some normal epithelial cells (FR-α Zimmerman, 1990;

Holm *et al.*, 1991, 1992, 1993; Ross *et al.*, 1994; Patrick *et al.*, 1997), hematological cells (FR-β and FR-γ; Shen *et al.*, 1994; Reddy *et al.*, 1999), and placenta (FR-β Ratnam *et al.*, 1989). However, it is highly overexpressed in a number of different cancers (Mattes *et al.*, 1990; Boerman *et al.*, 1991; Weitman *et al.*, 1992, 1994; Garin-Chesa *et al.*, 1993; Ross *et al.*, 1994; Toffoli *et al.*, 1997). For the most part, FR-α is overexpressed in carcinomas such as ovarian carcinomas, while FR-β is overexpressed in hematological cancers (Ross *et al.*, 1994). A wide variety of different therapeutic and diagnostic agents have been studied in folate-mediated targeting to malignant cells and tissues expressing FR (Leamon and Low, 1991; Leamon *et al.*, 1993; Lee and Low, 1995; Mathias *et al.*, 1996, 1998; Lu *et al.*, 1999). These studies were recently reviewed (Reddy and Low,1998; Wang and Low, 1998). Folate and its various conjugates are taken up by cancer cells by receptor-mediated endocytosis (Antony *et al.*, 1985; Leamon and Low, 1991; Turek *et al.*, 1993; Lee and Low, 1994). The route of internalization for the FR is to some extent still controversial. It is has been demonstrated that the FR becomes localized to non-clathrin-coated pits known as caveolae (Rothberg *et al.*, 1990; Turek *et al.*, 1993). Lending support to this mode of internalization, two specific inhibitors of caveolae assembly (nystatin) or internalization (phorbol-12-myristate) were shown to inhibit uptake of FA conjugates (Lee *et al.*, 1996), although neither drug inhibits clathrin-mediate endocytosis (Smart *et al.*, 1994). However, in other studies, folate receptors appear not to colocalize with caveolae (Mayor *et al.*, 1994; Wu *et al.*, 1997). Lending further support to the role of caveolae in folate uptake, FR chimeras targeted to clathrin-coated pits rather than caveolae were found to be unable to efficiently transport 5-methyltetrahydrofolate into the cytoplasm (Ritter *et al.*, 1995). Turek *et al.* (1993) showed that folic acid conjugates of BSA were taken up by caveolae and resided in multivesicular bodies at early time points (<60 min), but converged with the clathrin-mediate pathway used by transferrin conjugates at later times. However, clathrin-mediated endocytosis may be a relatively minor pathway for cells that overexpress very high numbers of the receptor (Rijnboutt *et al.*, 1996). A diagram indicating the various possible routes of uptake for both vitamin and growth factor receptors is given in Fig. 2. The final destination may be dependent on the multiplicity of the interaction between the targeted molecule and the receptor, where cross-linking of the receptors leads to uptake into caveolae (Mayor *et al.*, 1994). Due to the multiple copies of the folic acid conjugated to the liposome surface, liposomes may favor targeting to caveolae. Despite the large volume of work on the subject, a definitive answer as to the complete route of internalization following

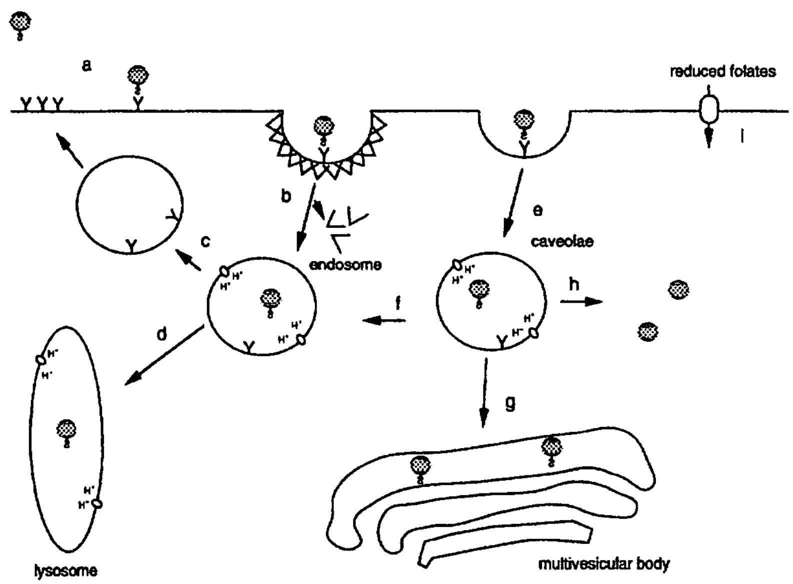

FIG. 2. Potential fates of ligand-targeted liposomes following binding to a target cell. Upon binding to cell-surface receptors, liposomes can either remain bound at the cell-surface, disassociate from the receptor, or accumulate in coated or noncoated invaginations. Following clathrin-mediated endocytosis (a), liposomes can be delivered to lysosomes (c) where they and their contents may be degraded by lysosomal peptidases and hydrolases. Receptors may be recycled back to the cell surface (b) or targeted for degradation in the lysosome (c). Some GPI-anchored receptors, such as the FR, can be taken up by caveolae-mediated endocytosis. Following internalization, the conjugated molecule can remain in caveolae, be transported to multivesicular bodies (g) or into the cytoplasm (h) and possibly reenter the lysosomal directed pathway (f) A substantial proportion of the folate-targeted molecules or liposomes appear to remain in a nondegradative compartment, allowing greater feasibility for delivering labile molecules *via* this route.

receptor binding is not known. An answer to this question may prove very important when selecting various molecules for encapsulation or complexation to lipid-based carriers.

Since FR-α is a GPI-anchored protein, phospholipase C treatment of FR-α-overexpressing cells results in a considerable loss of uptake of FA conjugates (Leamon and Low, 1992). In addition to the folate receptor, other receptors commonly found in caveolae include receptors for platelet-derived growth factor (PDGF receptor) (Liu *et al.*, 1996), bradykinin (de Weerd and Leeb-Lundberg, 1997), insulin (Goldberg *et al.*, 1987), and epidermal growth factor (EGFR; Mineo and Anderson, 1996). An extremely important advantage of targeting to this endocytic

pathway as compared to clathrin-mediated endocytic pathway is that the final destination of the targeted molecules in clathrin-mediated endocytosis is the lysosome, where degradative enzymes can degrade labile drugs, genes, or other biomolecules. The caveolae pathway appears to be rather nondestructive, as molecules are able to remain intact for up to days following binding to internalizing receptors (Leamon and Low, 1991; Wang *et al.*, 1995). This results in a significant enhancement of the activity for the delivered molecule (Leamon *et al.*, 1992; Rui *et al.*, 1998). In addition, similar to clathrin-coated vesicles, caveolae are also acidified to a relatively low pH (Lee *et al.*, 1996), allowing for the development of pH-sensitive liposomes for enhanced cytoplasmic delivery (Lee and Huang, 1996; Reddy and Low, 1998; Rui *et al.*, 1998).

B. Folic Acid as a Targeting Ligand: Coupling to Liposomes

Targeting of liposomes to folate receptors was demonstrated by using their natural ligand, folic acid, as a targeting moiety (Lee and Low, 1994). In order to act as liposome-targeting ligands folic acid molecules must be stably conjugated on the outer surface of the liposome in a way that does not impair receptor-binding properties of FA and allows unhindered interaction of the liposome-conjugated FA with FR. Ligands are attached to liposomes by conjugation to lipid molecules sufficiently hydrophobic to act as "membrane anchors" staying in the environment of hydrocarbon chains of the lipid bilayer rather than in the aqueous environment outside the liposome. Anchors with two closely positioned C_{16}–C_{20} alkyl or acyl chains, such as diacylglycerol derivatives, satisfy this requirement. Opposite to this hydrophobic domain, lipid anchor molecules usually have a chemically reactive group, such as primary amino, carboxy-, or thiol group, that can form a stable bond with the ligand (for review, see Park *et al.*, 1997c; Allen *et al.*, 1997). Distearoyl phosphatidylethanolamine and its N-derivatives are molecules of choice for many liposome–ligand conjugations. These lipid anchors are included in the liposome lipid composition before ligand conjugation or conjugated to ligands to form membranotropic conjugates that are later used to form ligandoliposomes. The molecule of folic acid has two carboxyl groups in its glutamate domain, of which the group in the γ-position can be modified without the loss of FR binding and has been used for making various FR-reactive conjugates, including liposomes. Folic acid has been coupled to preformed liposomes *via* a simple lipid anchor or to a lipid anchor with a PEG spacer having terminal primary amino group using water-soluble

carbodiimide EDAC (Lee and Low, 1994). In another approach, the conjugate was first synthesized and admixed into the lipids prior to the formation of the liposomes (Lee and Low, 1995). One of these coupling strategies is shown in Fig. 3. In this method, folate is first conjugated to diamino-polyethylene glycol using dicyclohexylcarbodiimide (DCC). In this reaction 70–80% of the conjugate contains the linker at the γ-carboxyl group; the remaining conjugate, however, contains the linker in the α-position and is not reactive with FR. These two components can be separated by ion exchange chromatography due to the difference in p*K*s between the α- and γ-carboxyl groups of the folate (p*K* 2.8 vs 4.5, respectively) (Fan *et al.*, 1991); however, the presence of the α-derivative does not seem to have negative effect on the targeting properties of subsequently produced FA-conjugated liposomes. The monoderivatized PEG was isolated and reacted with the hydrophobic "anchor" *N*-succinyl-distearoylphosphatidylethanolamine using a similar conjugation step. If the presence of α-conjugated folate is unacceptable, the conjugate can be constructed by stepwise addition of first, protected glutamic acid, and then, pteroic acid to the activated lipid-PEG linker (Knepper *et al.*, 1990), but at a higher cost and with more effort. The liposome lipids and the conjugate were mixed in an organic solvent; following the evaporation of the solvent, they were hydrated together in an aqueous buffer and subjected to usual steps of dispersion and extrusion through nanoporous track-etched membranes to produce 100-nm unilamellar vesicles . This allowed for a more efficient coupling process, avoided the difficulty of developing rigorous and reproducible coupling methods using chemically activated hydrophobic linkers in aqueous solutions, and also eliminated the exposure of drug-loaded liposomes to potentially deleterious coupling reagents and conditions. Finally, one may envision even more elegant methods for the conjugation of folic acid and other water-soluble vitamins to preformed, drug-loaded liposomes based on the remarkable ability of hydrophobically modified poly(ethylene glycols) to merge spontaneously their hydrophobic domains into the liposome bilayers without liposome destruction or even permeabilization (Uster *et al.*, 1996). Indeed, the usefulness of this "insertion" method was recently demonstrated for preparation of peptide- and oligosaccharide-linked liposomes (Zalipsky *et al.*, 1998) as well as for even larger ligands such as antibodies (Ishida and Allen, 1999) and antibody fragments (Kirpotin *et al.*, 2000b). One advantage of using a small, naturally occurring molecule such as folic acid for ligand-mediated targeting, compared to a protein such as an antibody or antibody fragment, is the relatively higher shelf life of the conjugate. Proteins are often more sensitive to changes in environmental conditions

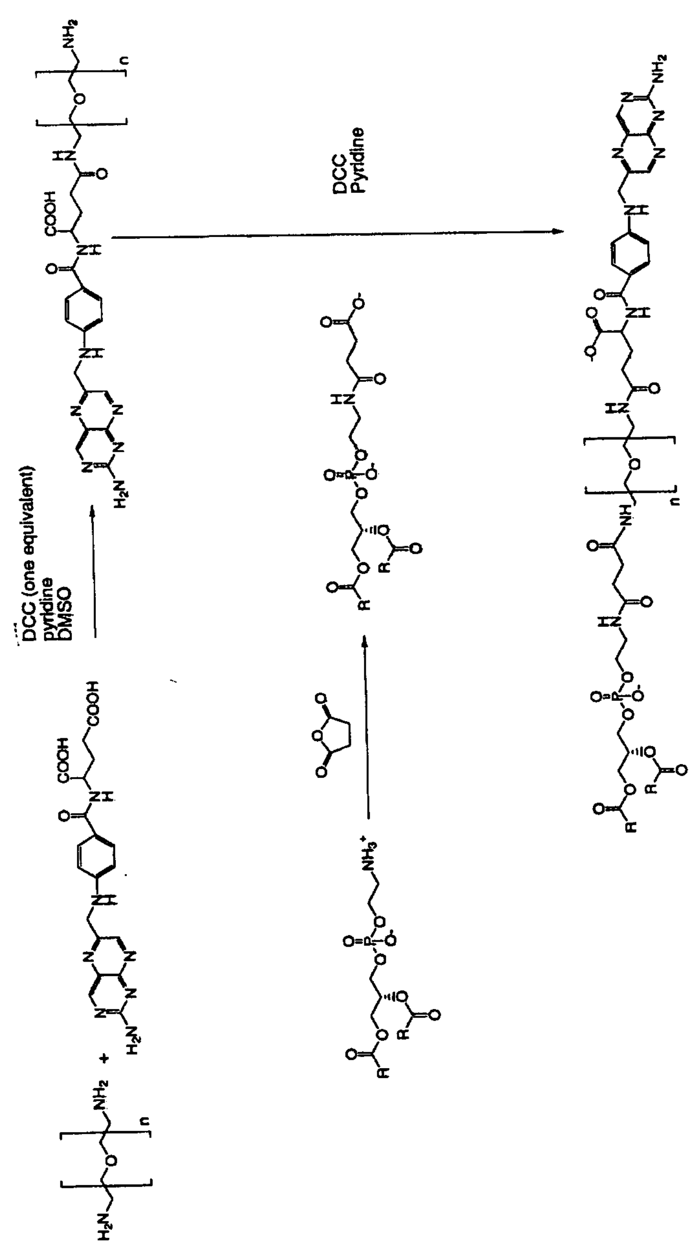

FIG. 3. Synthetic scheme for coupling of folic acid to a lipid anchor (1,2-distearoyl-3-*sn*-phosphatidylcholine; DSPE) *via* a poly(ethylene glycol) linker. Folic acid is first coupled to poly(ethylene glycol) bis-amine using dicyclohexylcarbodiimide as a coupling reagent. The monoderivatized folic acid-PEG conjugate is purified and subsequently coupled to *N*-succinyl-DSPE using a similar coupling reaction. *N*-succinyl-DSPE can be readily formed from DSPE and succinic anhydride in the presence of a weak organic base (Kung and Redemann, 1986).

and there may be significant stability concerns if they are stored for long periods at 4°C, conditions under which drug-loaded liposomes are normally stored.

C. Interaction of Folate-Targeted Liposomes with FR-Overexpressing Cells

Folate-targeted liposomes were first prepared by conjugation of folic acid to a lipid anchor already present in liposome membranes (Lee and Low, 1994, 1995). The PEG spacer, linking FA to the lipid anchor, was determined to be essential in distancing the targeting ligand from the liposome surface to allow binding to the folate receptor on the surface of the cancer cells (Lee and Low, 1994). Little or no cell association was seen when any of several short spacers were used to conjugate folic acid to the surface of the liposome. However, we have noted that while the liposomes with short-spacer FA conjugates did not bind to FR-overexpressing cells, they were quite capable of binding the milk folate-binding protein, which is essentially an extracellular domain of the folate receptor shed from the cell membranes (Kirpotin and Kolhouse, 1993, unpublished observation). Apparently, certain length and flexibility of the liposome–ligand spacer was necessary to allow unhindered binding of the liposome-tethered folic acid to the cell surface receptor; the same may be true for other small-molecule ligands as well. Gabizon and co-workers (1999) showed that the length of the spacer was also important when using FA-PEG-DSPE conjugates on the liposomes containing also unmodified PEG-DSPE used to increase their circulation lifetimes. Cell association was markedly increased when folate was conjugated to liposomes using a PEG spacer greater in length than the PEG chain of the more abundant unmodified conjugate (Fig. 4). Thus, it appears important for the folic acid to be extended above the polymer coat of the liposome to reduce steric hindrance to its receptor and to benefit from the conformational flexibility of the polymer linker which may allow its better access to cell membrane receptors.

Lee and Low (1994) studied the kinetics of folate–liposome associations with the cells. When targeted to FA-α-overexpressing KB cells, FA-derivatized liposomes were seen only at the cell periphery at early times, but were seen throughout the cytoplasm as a punctate fluorescence pattern at later times (4 h). Both the kinetics of internalization and the total number of liposomes bound at saturation (2.5×10^5) were lower than for the much smaller albumin conjugates studied in an earlier work (Leamon and Low, 1991). The authors suggested the latter may be a result of the multivalent nature of FA display when present

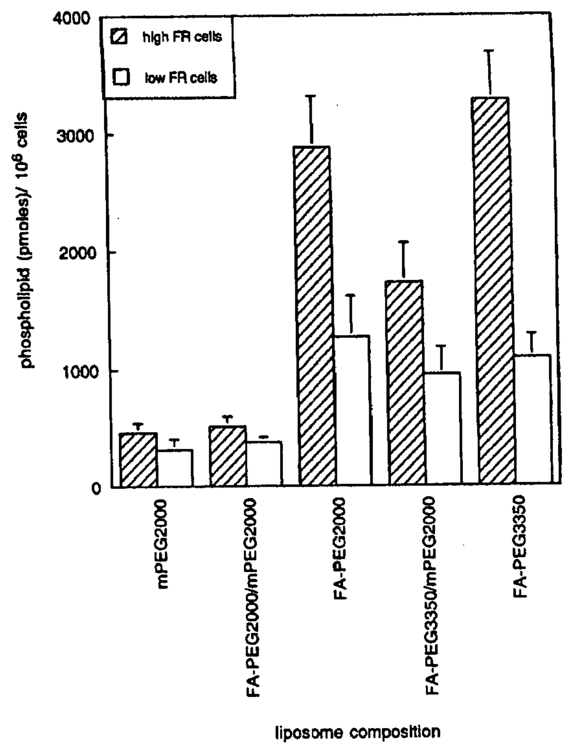

Fig. 4. Effect of PEG length on binding of FA-derivatized liposomes to KB cells. Liposome binding was determined by measuring the amount of cell associated [^{3}H]Chol. Liposomes were incubated with KB cells having low (low FR) or high either low- or high (high FR)-folate receptor expression. High expression of folate was achieved by passaging the cells in folate-free medium (the only source of folate being endogenous folate in fetal calf serum, added to the medium at 10%). Liposomes contained folate-PEG-DSPE conjugates with PEG having molecular weights of either 2000 or 3350 (PEG2000 and PEG3350) and in either the presence or absence of PEG2000-DSPE at approximately 7% of total liposomal phospholipids. The cells were incubated with liposomes for 24 h at 37°C. From Gabizon *et al.* (1999), with permission from the American Chemical Society.

in the form of liposomes. The depressed rate of internalization may result from an increased steric hindrance to cell surface receptors due to the larger size of the liposome, compared to albumin. FA liposomes were also shown to be endocytosed by looking at the difference in acid-removable liposomes following incubations at both 4° and 37°C (Lee and Low, 1994). While FA liposomes incubated with KB cells at 4°C could be quantitatively removed from the cells by an acid wash, only

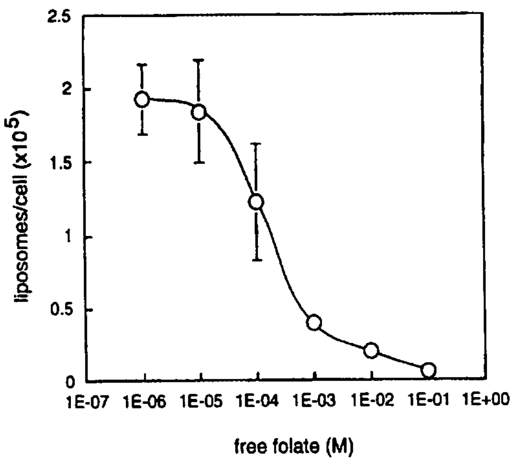

FIG. 5. Effect of folic acid on the uptake of folate-conjugated liposomes by KB cells. KB cells were incubated with calcein-loaded FA-derivatized liposomes for 4 h at 37°C in the presence of varying concentrations of free FA. The amount of cell-associated liposomes was determined from the calcein fluorescence in the detergent lysates of thoroughly rinsed cells. From Lee and Low(1994) with permission from ASBMB.

half of the liposomes could be removed following incubation at 37°C for 4 h. It is interesting that there are still a relatively high number of liposomes at the cell surface even at 4 h, a time where other receptors have been shown to be almost completely internalized to an intracellular localization. This may be a result of the different trafficking pathways involved in caveolae-mediated endocytosis compared to the more typically studied clathrin-mediated endocytosis, which terminates in the lysosome.

This association was shown to be specific for folate-mediated targeting by competitive inhibition studies with either free FA or antiserum against FR-α (Lee and Low, 1994). The inhibition of FA-derivatized liposome association with KB cells by free FA is shown in Fig. 5. Interestingly, a concentration approaching 1 mM free FA was required to inhibit FA-derivatized liposome uptake by KB cells (Fig. 5). This is in contrast to the relatively low K_d of free FA for the FR (0.01–1 nM; Kamen *et al.*, 1988) and the significantly lower concentrations of free FA (<200 nM) required to inhibit uptake of FA–deferoxamine conjugates (Wang *et al.*, 1996). The multivalent binding of liposome bound conjugates likely leads to this reduced inhibition and gives rise to a targetable therapeutic that may be useful *in vivo* when considering the relatively low free FA concentrations in plasma (<20 nM; Antony *et al.*, 1985; Kamen *et al.*, 1988; Antony, 1992).

D. Delivery of Antineoplastic Drugs to Cancer Cells by Folate-Targeted Liposomes

Several studies have reported the cytotoxicity of drug-loaded FA-targeted liposomes to FR-overexpressing cancer cells (Lee and Low, 1995; Rui *et al.*, 1998). Folate-mediated targeting of doxorubicin-loaded liposomes was shown to increase the cytotoxicity of doxorubicin to FR-overexpressing KB cells when compared to both free doxorubicin (1.6-fold) and nontargeted liposomal doxorubicin (45-fold; Fig. 6). The extrapolation of these results to *in vivo* conditions gives rise to even more promising results due in part to the favorable pharmacokinetic properties of liposomal carriers (Hwang, 1987; Allen *et al.*, 1995a; Drummond *et al.*, 1999). For example, although there is only a 1.6-fold increase in cytotoxicity in cell culture studies, the area under the concentration versus time curve for tumors is approximately 8-fold greater for liposomal doxorubicin compared to free doxorubicin, meaning there is a greater exposure of the cancer cells to the drug *in vivo* when encapsulated in liposomes (Unezaki *et al.*, 1995; Gabizon *et al.*, 1997). This should accentuate the favorable results seen in cell-culture studies even further.

Ara-C has been effectively delivered to KB cells using FA targeting of pH-degradable liposomes composed of di-C_{16}-plasmenylcholine (Rui *et al.*, 1998). The IC_{50} of ara-C was increased approximately 6000-fold when delivered in folate-targeted pH-degradable liposomes (0.49 μM) compared to free ara-C (2.8 mM). These liposomes are programmed to decompose at a pH characteristic of endosomal compartments. While the authors suggest the heightened sensitivity to ara-C is due in large part to the pH-sensitive lipid composition, it is also possible that targeting to what has already been shown to be a relatively nondestructive endocytic pathway contributes substantially to the increased cytotoxicity. This approach may prove especially fruitful for delivering readily degradable drugs or macromolecules.

E. Delivery of Genes and Antisense Oligonucleotides to Cells Using Folate Targeting

Folic acid has also been used as a ligand to target genetic material and antisense oligonucleotides to FR-overexpressing cells (Gottschalk *et al.*, 1994; Wang *et al.*, 1995; Lee and Huang, 1996; Reddy and Low, 1998). Folic acid-derivatized polylysine conjugates were used to condense DNA

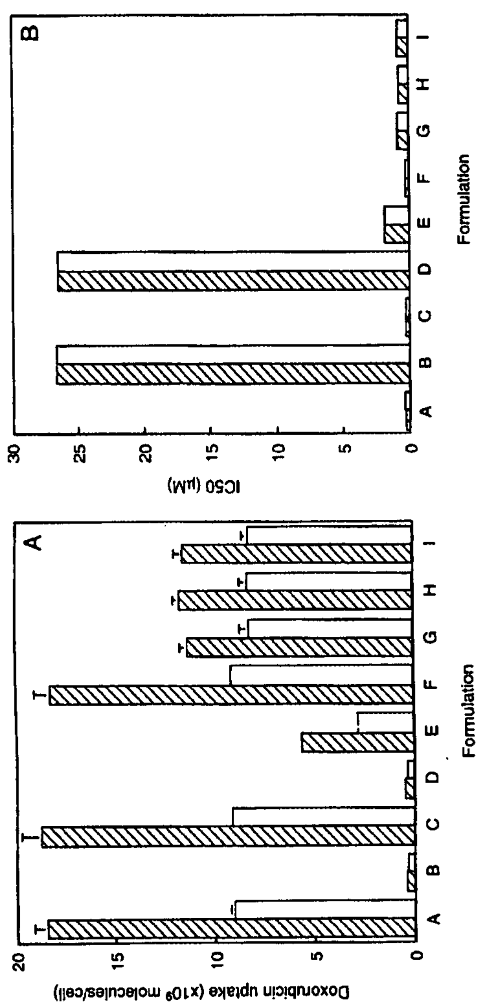

FIG. 6. Cellular uptake and cytotoxicity of folate-targeted and nontargeted formulations of doxorubicin. KB cells (cross-hatched bars) or HeLa cells (open bars) were incubated with doxorubicin formulation (100 µM DOX): folate-conjugated liposomal DOX without PEG coating (FA-L-DOX) (A); DOX in nontargeted, noncoated liposomes (L-DOX) (B); folate-conjugated PEG-coated liposomal DOX (FA-SSL-DOX) (C); nontargeted PEG-coated liposomal DOX (SSL-DOX) (D); FA-L-DOX + 1 mM folic acid (E); FA-L-DOX + 20 nM 5-methyltetrahydrofolate (F); free DOX (G); free DOX + "empty" folate-conjugated liposomes (H); free DOX + "empty" nontargeted liposomes (I). Cell-associated doxorubicin was determined by fluorescence spectroscopy, and cytotoxicity was determined using a tetrazolium (MTT) assay. IC_{50} refers to the concentration of doxorubicin that results in 50% decrease in viability compared to sham-treated control. Adapted from Lee and Low (1995), with permission from Elsevier Science.

and antisense oligonucleotides and deliver them specifically to tumor cells (Gottschalk *et al.*, 1994; Citro *et al.*, 1992, 1994; Ginobbi *et al.*, 1997). Gene expression using these targeted complexes was shown to be dependent on the conjugated folate and was also markedly enhanced when coincubated with a replication-defective adenovirus, indicating the need for endosomal disruption for efficient release of DNA from intracellular organelles (Gottschalk *et al.*, 1994).

Lee and Huang (1996) developed a pH-sensitive lipid–DNA particle composed of polylysine-condensed DNA, DOPE, and the pH-sensitive lipid cholesteryl hemisuccinate (CHEMS). At low lipid-to-polylysine condensed DNA ratios the complexes were positively charged and gene expression was independent of folate targeting. However, at higher ratios, where the complexes obtained a net negative charge and a relatively small size (~74 nm), folate targeting resulted in a 20- to 30-fold increase in transfection efficiency when compared to cationic lipid–DNA complexes (DC-Chol:DOPE) and were significantly less cytotoxic. A similar approach complexed polylysine-condensed DNA with FA-targeted anionic liposomes containing the "caged" pH-sensitive lipid *N*-citraconyldioleoylphosphatidylethanolamine (*N*-cit-DOPE; Reddy and Low, 1998). This "caged" lipid was originally described by Drummond and Daleke (1995) and later shown to be useful in preparing pH-sensitive liposomes (Drummond and Daleke, 1997). The use of this "caged" *N*-cit-DOPE was shown to increase transfection efficiency by a factor of 4 to 5 when compared to the similar composition described by Lee and Huang (1996). Other folate-targeted cationic lipid based delivery vehicles may enhance transfection efficiencies even further, similar to the results seen with anti-HER2 targeted cationic lipid–DNA complexes (Section III,E). For lipid-based gene delivery vehicles, active targeting via receptors, such as the folate receptor.

Antisense oligonucleotides against the EGFR were delivered to KB cells via folate targeting to cultured KB cells (Wang *et al.*, 1995). The liposomes utilized in these experiments were composed of eggPC, cholesterol, and either with or without folate-PEG-DSPE. A fluorescein-labeled oligonucleotide was used to show a 16-fold increase in uptake over free oligonucleotides and a 9-fold increase over nontargeted liposomal oligonucleotide. Uptake could be inhibited by free folic acid (1 mM), demonstrating the specificity of internalization. However, delivery to the nucleus was relatively inefficient, likely due to the relatively stable lipid composition used in this particular formulation and thus inability to escape the confines of the endosomes or lysosomes. Finally, while folate-specific growth inhibition did occur, there was no significant difference in growth inhibition between the anti-EGFR oligonucleotide

and the scrambled sequence. These results suggest that folate targeting may be effective at delivering both oligonucleotides and plasmid DNA to receptor-overexpressing cells. However, the efficiency of delivery, both from the endosome and under *in vivo* conditions, needs to be increased and studied further.

F. *In Vivo* Implications for Folate-Mediated Liposome Targeting

To date, folate targeting of liposomes has been almost exclusively studied in cell culture, with minimal work being completed *in vivo*. Mathias and co-workers (1996, 1998, 1999) have used folic acid to target small-molecule radiotracers for imaging folate receptor-overexpressing tumors *in vivo*. However, the pharmacokinetics of small-molecular-weight compounds such as these are undoubtedly different from those of sterically stabilized or even conventional liposomal carriers. For example, these relatively small-molecular-weight radioconjugates are found in high amounts in the kidney, an organ that would be relatively inaccessible to large liposomal carriers approximating 100 nm in size.

An important concern for use of a folate receptor targeting approach *in vivo* involves the toxicity to normal healthy tissues, specifically those of hematopoietic lineage. The β-isoform of the folate receptor (FR-β) is expressed in high levels on hematopoietic cells (Ross *et al.*, 1994; Shen *et al.*, 1994; Reddy *et al.*, 1999), and thus targeting of cytotoxic agents to this receptor may be expected to result in significant bone marrow toxicity. Fortunately, Reddy and co-workers (1999) were able to demonstrate that CD34+ hematopoietic cells do not bind FA-conjugated liposomes, although these cells overexpress FR-β.

The final potential problem with targeting to cell-surface receptors using FA-conjugated liposomes is the binding-site barrier phenomenon observed for tumor-specific antibodies (Juweid *et al.*, 1992; Fujimori *et al.*, 1990): the binding of FA conjugates to FR-α at the site of liposome extravasation may limit its penetration of the tumor and thus accessibility to cancerous cells located any significant distance from the supporting vasculature. However, limited diffusion and the increased bioavailability of encapsulated drugs may be enough in itself to provide a significant improvement to nontargeted liposomes. An alternative approach may be to use antibody fragments against the folate receptor rather than use the ligand itself. The reduced avidity of the targeting ligand for the receptor may result in a more even tumor distribution of the carrier, similar to that seen with anti-HER2-targeted immunoliposomes (Section III,D). While speculation on the feasibility of targeting FRs *in vivo* suggests both significant promise and a degree of uncertainty, the true test lies in the completion of efficacy studies in appropriate animal tumor models, such as in the treatment of ovarian cancers.

G. Other Vitamin Receptors as Targets for Ligandoliposomes

Few data are available on the targeting of liposomes to cells using receptors for vitamins other than folate. However, the successful development of folate targeting laid the methodological groundwork for the use of other vitamins as ligands for liposomal delivery. As the receptors of these vitamins become better understood with respect to routes of internalization and expression patterns on both normal and malignant cells, further development of other vitamin-targeted strategies will likely be attempted. At present, pyridoxine, pyridoxal phosphate, biotin, riboflavin, and nicotinamide have been reported as targeting ligands other than liposomes for therapeutic or diagnostic agents (Low *et al.*, 1997a,b; Holladay *et al.*, 1999). The developed conjugation strategies can be easily adapted for conjugation of these ligands to liposomes. Preparation of fully functional conjugates of cyanocobalamine (B_{12}) with radioiodinated markers and spacer molecules was recently reported, and the chemistries appears amenable to liposome conjugation (Wilbur *et al.*, 1999; McEwan *et al.*, 1999).

Aside from specific targeting of their receptors, vitamins have been used in distinct roles in the development of liposome technology. Thus, biotin has been used as an adaptor molecule for conjugating targeting ligands to liposomes via a noncovalent biotin–streptavidin linkage (Rosenberg *et al.*, 1987; Harasym *et al.*, 1995; Wong *et al.*, 1997). α-Tocopherol (vitamin E) has been used to prevent oxidative damage to liposomes during storage (Barenholz *et al.*, 1993). These functions of vitamins in liposome technology are out of the scope of this chapter. However, it is safe to say that the groundwork for the use of vitamins as liposome-targeting ligands in the clinic has been firmly set and awaits a promising future.

III. Targeting Liposomes to Growth Factor Receptors

A. Growth Factor Receptors as Recognition Molecules of Malignant Cells

Like their effectors, hormone receptors present a family of structurally and functionally different proteins. Some hormone receptors, such as steroid receptors, are intracellular proteins that are inaccessible

for targeting. Steroid hormones may reach their receptors following diffusion across biological membranes. Receptors for other hormones, such as peptide hormones and growth factors that do not permeate the cell membrane, are plasma membrane proteins whose effector-binding domains are exposed on the cell surface. It is these accessible receptors that prove most valuable for cell-targeted delivery of drugs. The epidermal growth factor receptor (EGFR), fibroblast growth factor receptor (FGF receptor), and HER2/neu receptor have all been shown to be overexpressed in different malignancies (Sporn and Roberts, 1985; Slamon *et al.*, 1987, 1989; Mansson *et al.*, 1989; Beitz *et al.*, 1992; Khazaie *et al.*, 1993; Fox *et al.*, 1994; Scher *et al.*, 1995). In addition, angiogenic vascular endothelial cells supporting the tumor overexpress vascular endothelial growth factor (VEGF) receptors (Shibuya *et al.*, 1990; Tischer *et al.*, 1991; De Vries *et al.*, 1992). The expression levels of these various receptors have been associated with tumor proliferation or metastasis (Yu *et al.*, 1991, 1994; Niehans *et al.*, 1993). Often, high expression levels of these receptors are associated with an increased risk of recurrence or poor survival (Berchuck *et al.*, 1990; Borg *et al.*, 1990; Toi *et al.*, 1991; Chrysogelos and Dickson, 1994; Fix, 1994; Press *et al.*, 1997). These receptors are attractive targets due not only to their high expression levels on malignant cells but also because they have been shown to be endocytosed by receptor-bearing cells following binding of their appropriate ligand or an antibody agonist (Pastan and Willingham, 1981; Tagliabue *et al.*, 1991; Sorkin and Waters, 1993; French *et al.*, 1994; Hurwitz *et al.*, 1995; Kirpotin *et al.*, 1997a). Thus, similarly to vitamin receptors discussed above, targeting to one of these receptors potentially allows the therapeutic agent access to an internal localization in what can be called a "Trojan horse" approach.

The HER2/neu receptor recently became an extensively studied target for liposomes and lipid-based gene- and drug-delivery systems. This receptor is a glycosylated transmembrane protein of approximately 185 kDa that possesses tyrosine kinase activity. It is a member of the epidermal growth factor receptor (EGFR) family and is involved in many growth-signaling pathways within the cell, alone or in cooperation with other members of EGFR family. Several excellent reviews have described the basic biology and biochemistry of the HER2/neu receptor (Hynes and Stern, 1994; Hung and Lau, 1999). This protein has high levels of overexpression in different malignancies (10^5–10^6 receptors/cell), low level of expression in healthy tissues, and homogeneous and stable expression in primary tumors and sites of metastasis (Press *et al.*, 1990; Lewis *et al.*, 1993; Niehans *et al.*, 1993). These characteristics made this protein a prime molecular target for cancer

immunotherapy using a recombinant humanized monoclonal antibody (trastuzumab) and has recently made its way into the clinic (Baselga *et al.*, 1999; Pegram *et al.*, 1998; Shak, 1999). These properties are also likely responsible for the many encouraging preclinical results obtained with an anti-HER2-targeted immunoliposome carrying encapsulated doxorubicin (Section III,D).

B. Design of HER2-Targeted Immunoliposomes

In contrast to the folate receptor targeting described above, where the natural ligand for FR was also the liposome-targeting ligand, targeting of liposomes to HER2 receptor exemplifies a different approach in which the targeting ligand is an antibody against extracellular portion of the receptor.

The history of antibody-targeted liposomes (immunoliposomes) encompasses two decades of research (Allen *et al.*,1997). Although immunoliposomes or, in fact, any ligand-targeted liposomes have not yet made their way to the clinic, one can put forward a set of criteria for a successful practical design of immunoliposomes as cancer drug-delivery vehicles (Table II). As discussed in the preceding section, growth factor receptors and especially HER2/neu satisfy the criteria for target antigen selection quite well. Evidently, the targeting function of an immunoliposome requires only the presence of the antigen-binding domain, leaving a researcher with a choice of liposome-conjugated antibodies. A popular option of conjugating whole immunoglobulin molecules to liposomes appears suboptimal because of the poorly defined conjugation site, immunogenicity of xenogeneic IgG (Harding *et al.*, 1997), and enhanced blood clearance of immunoglobulin-conjugated liposomes mediated by mononuclear phagocyte Fc receptor (Aragnol and Leserman, 1986; Derksen *et al.*, 1988). The use of recombinant humanized anti-HER2 Fab′ fragments proved to be a better solution. Cysteine residues at the hinge region provided a unique conjugation site by efficient thiol-maleimide chemistry (Martin *et al.*, 1981; Martin and Papahadjopoulos, 1982). Similarly to folate-conjugated liposomes, when steric stabilization of immunoliposomes is desirable in order to prolong their circulation lifetime for better distribution into a tumor, PEG coating hinders the binding of an antibody to cell surface receptors if the spacer between the antibody and the lipid anchor is too short. Attachment of Fab′ fragments to the maleimide-activated terminal end of a liposome-linked PEG-lipid (PEG-DSPE) (Fig. 7) resulted in an unimpeded association of immunoliposomes with target cells while preserving long-circulating properties (Shahinian and Silvius, 1995; Zalipsky *et al.*,

Design Criteria for Ligand-Directed Liposome Targeting

Component	Considerations for optimum design
Target antigen	*Expression*
	Highly and homogeneously overexpressed in target tissue
	Function
	Vital to tumor progression, so that down-modulation does not occur or is associated with therapeutic benefit
	Shedding of antigen
	Limited, to avoid binding to soluble antigen and accelerated clearance
Targeting ligand	*Affinity*
	High enough to ensure binding at low liposome concentrations
	Low enough to avoid "binding-site barrier" effect (Weinstein)
	Immunogenicity
	Humanized MAb, to remove murine sequences. Use fragments without Fc portion (Fab′, scFv) to avoid interaction with Fc receptor
	Small molecular weight ligands should not be immunogenic—may act as haptens
	Internalization
	Efficient endocytosis by target cells is desirable
	Production
	Easy and economical scale-up, e.g., by efficient bacterial expression system
	Stable during storage
Ligand–liposome linkage	*Stability*
	Covalent attachment to hydrophobic anchor, stable in blood
	Attachment site
	Away from the binding site to ensure correct orientation of antibody or ligand molecule.
	Well-defined, to ensure reproducibility and uniformity of coupling
	Avoids steric hindrance (e.g., from PEG) of ligand binding and internalization
	Chemical nature of the linker
	Nontoxic, nonimmunogenic, and avoids opsonization
	Does not affect drug loading or membrane stability
	Excess linker may be quenched to avoid nonspecific coupling to biomolecules
	Good availability, economical manufacturing process
Liposome	*Stability*
	Stable as intact construct *in vivo*

(*continued*)

Table II (*continued*)

Component	Considerations for optimum design
	Pharmacokinetics
	Long circulating
	Tumor penetration
	Capable of extravasation in tumors
	Small diameter improves penetration into tumor tissue
Drug	*Encapsulation*
	Efficient, high capacity (e.g., by remote loading)
	Encapsulated drug storage-stable and resists leakage
	Bystander effect
	Drug affects tumor cells not directly targeted (bystander cells)
	Interaction with target cells
	Effective against target cell population
	Cytotoxicity enhanced by binding of ligand

1996, 1998; Kirpotin *et al.*, 1998). An even better choice of liposome-targeting antibody may be single-chain Fv (scFv) fragments selected from phage-display libraries (Marks *et al.*, 1992; Schier and Marks, 1996). Single-chain Fvs are constructed from human immunoglobulin sequences and thus are likely to have little or no immunogenicity in humans. Phage-display selection and affinity maturation (Schier and Marks, 1996; Schier *et al.*, 1996) allow construction of scFvs with a broad range of specificities and binding characteristics. Single-chain Fvs are relatively small (26–29 kDa) recombinant proteins that can be produced in quantities in bacterial hosts, are easily purified, and can be engineered to contain a unique liposome conjugation site, for example, a C-terminal cysteine group, away from the antigen-binding site. Last but not least, a recently reported scFv selection technique based on phage internalization into live target cells (Becerril *et al.*, 1999) allows the creation of scFvs optimized not only for selective binding, but also for selective internalization by the target cells, which is a desirable component of liposome targeting (Nielsen *et al.*, 2000).

Anti-HER2 immunoliposomes targeted to HER2-overexpressing tumors were a subject of several recent studies (Goren *et al.*, 1996; Park *et al.*, 1995, 1997a,1997b, 2000; Kirpotin *et al.*, 1997a, 1997b, 1998, 2000a). In these studies anti-HER2 immunoliposomes were constructed on the platform of sterically stabilized PEG-coated liposomes containing doxorubicin encapsulated by the ammonium ion gradient method. These liposomes are similar to liposomal doxorubicin, which has been recently introduced into the clinic under the trade name of Doxil (Alza Corp.) (Martin, 1998; Gabizon and Barenholz, 1999).

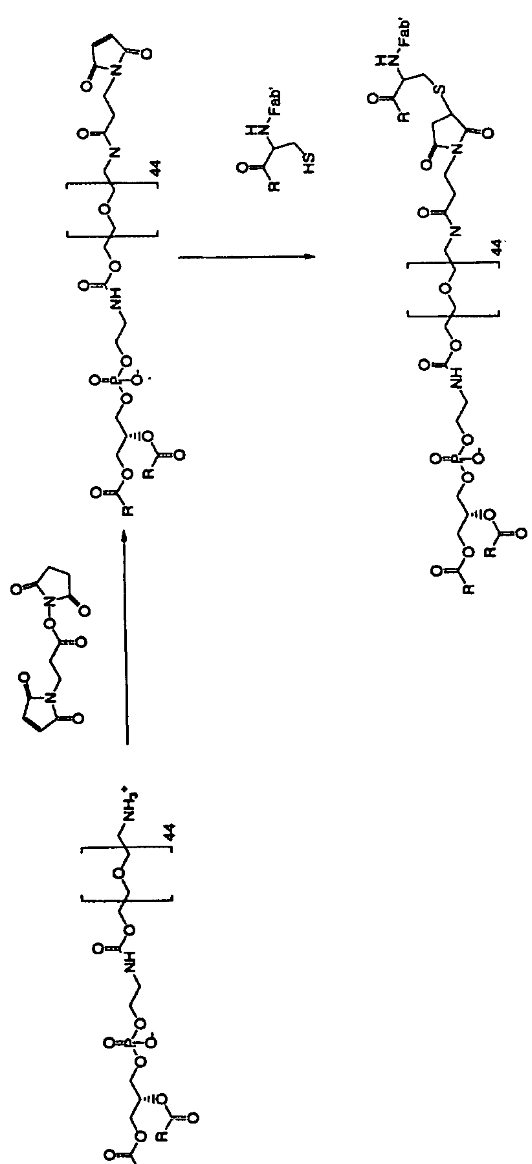

FIG. 7. Synthetic scheme for conjugation of Fab′ fragments to liposomes. An amino-PEG-DSPE conjugate is derivatized with the bifunctional cross-linking reagent, *N*-(γ-maleimidopropionyloxy)-succinimide The obtained maleimide-terminated PEG-DSPE derivative can be incorporated into liposomes and subsequently derivatized with a Fab′ or scFv fragment via reduced sulfhydryl group of peptide terminal cysteine, located away from the antigen-binding site specific for a cell-surface receptor. Alternatively, the fragments can be conjugated with maleimido-PEG-DSPE in solution and then captured into the liposome bilayer by co-incubation of the liposomes with the conjugate (Kirpotin *et al.*, 2000a).

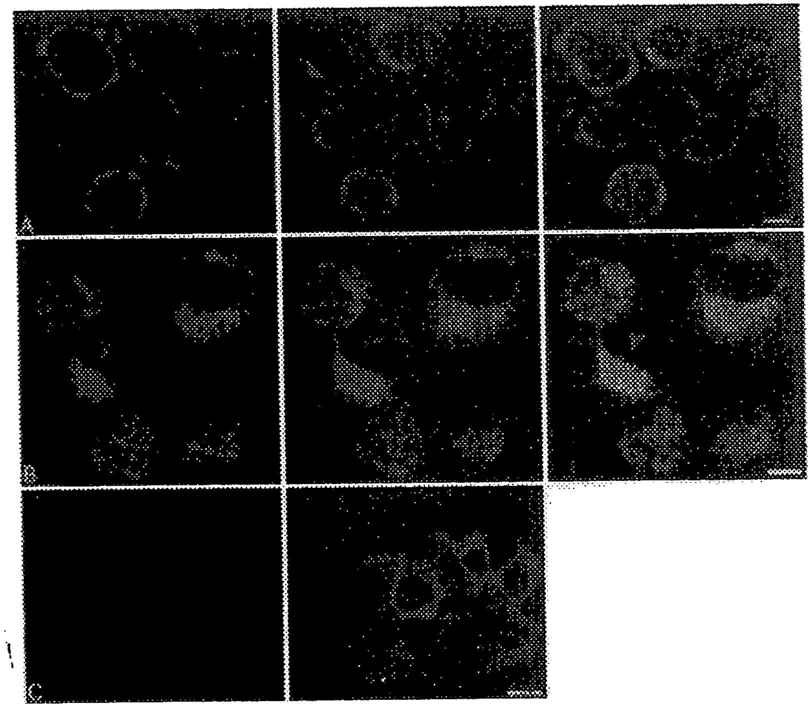

FIG. 8. Uptake of rhodamine-labeled antiHER2 immunoliposomes (left column) and fluorescein-labeled transferrin (middle column) by HER2-overexpressing SKBR3 cells (A,B) or by MCF-7 cells having low expression of HER2 (C). The cells were incubated with liposomes at 0.1 mM of liposome phospholipid and 37°C for 10 min (A) or 30 min (B and C). Liposome localization was visualized by confocal microscopy of intact cells. Superimposed images are shown in the right column. From Kirpotin *et al.* (1997) with permission from the American Chemical Society.

C. *In Vitro* Studies with Anti-HER2 Immunoliposomes

Interaction of anti-HER2 sterically stabilized immunoliposomes with target cancer cells was first studied in the cultures of human breast carcinoma cells of either low (MCF-7; 10^4 receptors/cell) or high (SKBR-3; 10^6 receptors/cell) expression levels of the HER2 receptor. Confocal fluorescence microscopy showed colocalization of anti-HER2 targeted immunoliposomes and fluorescein-labeled transferrin (Fig. 8), indicating uptake by HER2-overexpressing cells of liposomes into the clathrin-mediated endocytic pathway (Park *et al.*, 1995; Kirpotin *et al.*, 1997a). MCF-7 cells expressing low levels of the HER2 receptor were able to

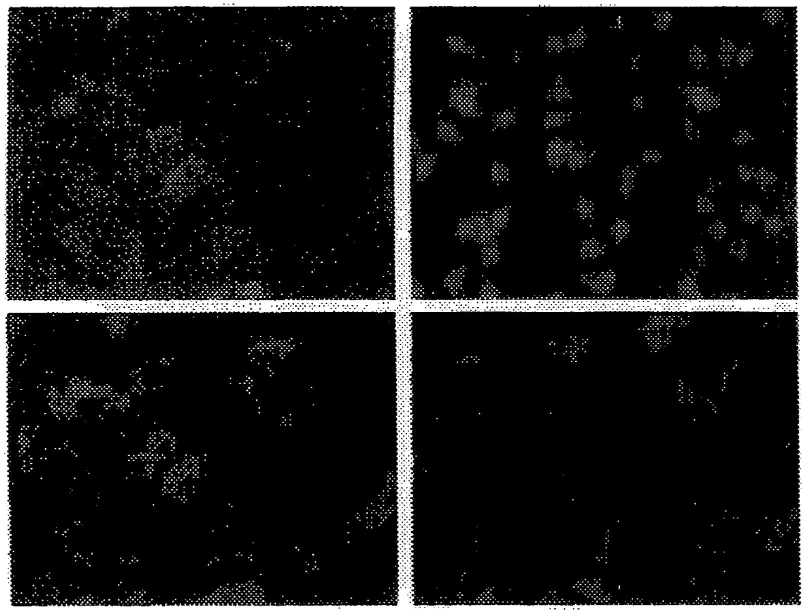

FIG. 9. Intracellular distribution of the components of PEG-DSPE-stabilized cationic liposome (DOTAP:DOPE:PEG-DSPE) complex with FITC-labeled phosphorothioate oligonucleotide (PS ODN) in HER2-overexpressing SKBR3 cells. (Top row) FITC-labeled PS ODN; (bottom row) rhodamine-labeled lipid. Only the complexes where a portion of PEG chains was conjugated to the anti-HER2 Fab′ (left column) show nuclear delivery of the oligonucleotide. (Right column) Nontargeted complexes. From Meyer *et al.* (1998), with permission from ASBMB.

take up the transferrin conjugate but not the immunoliposomes. Similar results were obtained with anti-HER2-targeted liposomes containing cationic lipids and complexed with antisense oligonucleotides (Meyer *et al.*, 1998). In this case, not only were the targeted complexes endocytosed, but only those containing anti-HER2-conjugated Fab′ were able to effectively deliver the oligonucleotide payload to the cell nuclei (Fig. 9). Both binding and endocytosis of anti-HER2-targeted immunoliposomes depended on the number of conjugated anti-HER2 Fab′, which reached a plateau at approximately 30–40 Fab′ per 100 nm liposome (Kirpotin *et al.*, 1997a). At 0.025 mM of liposome phospholipid in the cell growth medium, liposome uptake reached a maximum of 8,000–25,000 liposomes per cell after 3–4 h of incubation. Under these conditions, more than 80% of the liposomes were endocytosed. Uptake by low-HER2-expressing MCF-7 cells was undetectable.

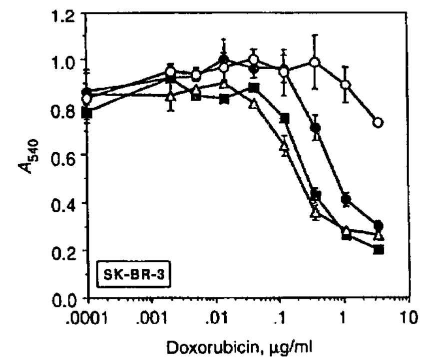

FIG. 10. *In vitro* cytotoxicity of the free or liposome-encapsulated doxorubicin in SKBR3 cells. The cells were treated with doxorubicin in anti-HER2 immunoliposomes without PEG coating ("conventional" immunoliposomes) (●), PEG-coated anti-HER2 immunoliposomes (○), PEG-coated immunoliposomes conjugated to an irrelevant Fab′ (■), or free doxorubicin (□). From Park *et al.* (1995) with permission. © 1995 by the National Academy of Sciences.

Cytotoxicity experiments were performed with doxorubicin-loaded anti-HER2 immunoliposomes (Park *et al.*, 1995; Fig. 10). In these initial experiments, the antibody was conjugated directly to the liposome surface rather than to the distal end of a PEG spacer. These targeted immunoliposomes were shown to be as cytotoxic to HER2-overexpressing SKBR-3 cells as free doxorubicin ($IC_{50} = 0.3\,\mu g/ml$). Nontargeted liposomes or liposomes containing an irrelevant antibody were shown to be significantly less toxic than either anti-HER2 immunoliposomes or free doxorubicin. Liposomes containing the sterically hindering PEG-DSPE were shown to also reduce the cytotoxicity of the targeted formulation, presumably due to an interference of receptor binding by the conjugated polymer. Targeted immunoliposomes were also relatively noncytotoxic to HER2 negative lung fibroblast cells (WI-38). Binding of the immunoliposomes to the HER2 receptor does not ensure endocytosis. Liposomes conjugated to a HER2-specific antibody that did not induce internalization were only as cytotoxic as the nontargeted liposomal formulation (Goren *et al.*, 1996). This indicates how essential endocytosis is to increasing the effectiveness of doxorubicin delivery into target cells.

D. *In Vivo* Studies with Anti-HER2 Immunoliposomes

In vivo pharmacokinetic and antitumor efficacy studies in xenograft models have yielded very promising results using anti-HER2 immuno-

liposomes targeted by anti-HER2 Fab′ and scFv (Park *et al.*, 1997a,b, 1998, 2000; Kirpotin *et al.*, 1997b, 1998, 2000). Pharmacokinetic studies in rats demonstrated minimal differences in circulation lifetimes between nontargeted sterically stabilized liposomes and anti-HER2 immunoliposomes even after repeated weekly injections of immunoliposomes ($t_{1/2}$ ~16 h). This is important because antibody conjugation has been previously shown to significantly reduce the circulation lifetimes of liposomes when attached directly to the liposome surface (Debs *et al.*, 1987) or to the termini of liposome-conjugated PEG chains (Mori *et al.*, 1991; Allen *et al.*, 1994, 1995b; Goren *et al.*, 1996; Zalipsky *et al.*, 1996), especially after repeated administration (Harding *et al.*, 1997). Evidently, the use of Fab′ or scFv fragments for targeting eliminated unwanted interactions of extraneous IgG sequences with immune cells contributed to uncompromised pharmacokinetics of these immunoliposomes.

An interesting finding was that anti-HER2-targeted immunoliposomes did not accumulate in HER2-overexpressing human tumor xenografts to a greater extent than nontargeted sterically stabilized liposomes (Kirpotin *et al.*, 1997b, 2000). This was similar to the findings of Gabizon and co-workers, who developed a similar immunoliposome using a noninternalizable antibody for tumor targeting (Goren *et al.*, 1996). These results suggest that the rates of extravasation into the tumor and low rate of subsequent removal from the tumor control the accumulation of liposomes in the tumor and not active targeting. However, the distribution of liposomes within the tumor was more uniform compared to nontargeted SSL and was often found within the cancer cells themselves (Kirpotin *et al.*, 1997b, 2000), similar to that seen in HER2-overexpressing cells in culture (Kirpotin *et al.*, 1997a). Nontargeted sterically stabilized liposomes were most often found within tumor-resident macrophages. The greater penetration and more uniform distribution may be a result in part of the relatively low avidity of immune fragments compared to full antibodies. This may help partially overcome the binding site barrier resulting from tight antibody binding to receptors located at the surface of the tumor (Weinstein *et al.*, 1987; Fujimori *et al.*, 1990).

The favorable pharmacokinetics, tumor distribution, and endocytosis of anti-HER2 Fab′ and scFv-immunoliposomes helped translate into higher antitumor efficacy of anti-HER2 liposomal doxorubicin in xenograft models of HER2-overexpressing human breast cancers in immunodeficient mice (Park *et al.*, 1997a, 2000). Injections of HER2-targeted or nontargeted liposomal doxorubicin or free doxorubicin were started when tumor volumes reached 200–1000 mm^3 and continued for total of three weekly doses of 5 mg/kg. Antitumor efficacy was monitored by measuring the decrease in tumor size following start of treatment. Anti-HER2 immunoliposomes were shown to be more effective than saline control, free doxorubicin, or nontargeted SSL in reducing tumor size following this schedule of treatments (Table III). In addition a considerable number of tumors, up to 60%, completely regressed with no pathological evidence of tumor cells in the tumor inoculation site. Significant reduction in tumor size was achieved when the immunoliposome treatment was delayed until the tumor reached 1000 mm^3 in volume (Table III) (Park *et al.*, 2000). This is an unusual result for antitumor efficacy studies on xenografts, where treatment often starts at sizes equal to or less than 100 mm^3 due to the poor responsiveness of larger tumors to most chemotherapy. There were also no notable toxicities or weight loss in the nude mice treated with anti-HER2 immunoliposomes. Because the immunoglobulin with Fab portions identical to these liposome-conjugated Fab′ suppresses the growth of HER2-overexpressing tumors (Baselga *et al.*, 1999) and, in fact, is now used in the clinic against HER2-overexpressing breast cancers (trastuzumab, Herceptin), control experiments were performed with "empty" anti-HER2 immunoliposomes and the combination of trastuzumab IgG with nontargeted liposomal doxorubicin. However, both these treatments were less effective than doxorubicin formulated into anti-HER2 immunoliposomes (Park *et al.*, 2000). Superior antitumor activity of doxorubicin delivered in HER2-targeted immunoliposomes against HER2-overexpressing xenografts appears to be the result of their specific internalization by malignant cells in the tumor (Kirpotin *et al.*, 1997b, 1998, 2000) and the overall *in vivo* performance of anti-HER2 immunoliposomes, as the targeted drug carrier was related to the fulfillment of the criteria for ligandoliposome design laid out in Table II).

E. Targeted Delivery of Nucleic Acids to Cells through HER2 Receptors

Targeting gene delivery to malignant cells has also been shown to increase transfection efficiency using both viral (Goldman *et al.*, 1997; Boerger *et al.*, 1999; Gu *et al.*, 1999) and cationic lipid-based (Kao *et al.*, 1996; Kikuchi *et al.*, 1996; Park *et al.*, 1997b,c; de Lima *et al.*, 1999) gene-delivery approaches. Although cationic lipid-based gene delivery vehicles can be quite efficient *in vitro*, the need to modify the lipid compositions for increased stability *in vivo* also reduces their transfection competency. Modifying the complexes for specific targeting to growth factor receptors has been shown to help overcome some of this loss in

TABLE III
ANTITUMOR ACTIVITY OF VARIOUS DOXORUBICIN FORMULATIONS AGAINST BT-474 XENOGRAFTS IN NUDE MICE[a,b]

	Treatment started				Treatment outcome	
Treatment	At day	Tumor size, (mm^3)	At day	Tumor size, (mm^3)	p	Complete regressions/ total tumors
Study A						
Saline control	7	206 ± 25	43	4150 ± 2724		0/11
Ls-DOX	7	235 ± 38	43	425 ± 370		0/11
Anti-HER2 ILs-DOX (Fab′)	7	232 ± 24	43	105 ± 132	0.013 (vs Ls-DOX)	5/10
Anti-HER2 ILs-DOX (scFv)	7	203 ± 32	43	50 ± 33	0.006 (vs Ls-DOX)	6/11
Study B						
DOX + Trastuzumab	25	999 ± 387	56	5100 ± 4035		0/10
Anti-HER2 ILs DOX	25	1031 ± 371	56	263 ± 175	0.006	0/10
Study C						
Saline control	16	370 ± 58	43	3800 ± 1270		0/10
Ls-DOX + Trastuzumab	16	353 ± 29	43	783 ± 344		0/10
Anti-HER2 ILs DOX	16	338 ± 29	43	295 ± 147	0.003	3/10

[a] Adapted from Park *et al.* (2000).

[b] Animals were treated according to the following protocols (tumor inoculation at day 0). *Study A*: 0.125 mg of doxorubicin encapsulated in nontargeted sterically stabilized liposomes (Ls-DOX) or in anti-HER2 immunoliposomes (anti-HER2 Ils-DOX) injected intravenously at days 7, 14, and 21. *Study B*: 0.05 mg of doxorubicin solution (DOX) (maximum tolerated dose) or 0.125 mg of anti-HER2 ILs-DOX injected intravenously at days 25, 32, and 39; DXR group also received 75 μg of anti-HER2 antibody (Trastuzumab) intraperitoneally at days 25, 28, 32, 35, 39, and 42. *Study C*: 0.1 mg of doxorubicin as SL-DOX or as anti-HER2 Ils-DOX injected intravenously at days 16, 22, and 29; Ls-DOX group also received 7.5 μg of Trastuzumab antibody intraperitoneally at days 16, 19, 22, 25, 29, and 32. Saline controls: animals received injections of excipient (HEPES- or phosphate-buffered physiological saline) instead of the drug injections. Tumor size data are mean ±standard deviation; p, probability of the null hypothesis for the difference of the mean tumor volumes between drug treatment groups at the indicated day (by the independent t test). The number of complete regressions is calculated at day 56.

efficiency (Park *et al.*, 1997b,c). Condensed and PEG-stabilized cationic lipid–DNA complexes were shown to have a 20-fold increase in reporter gene expression following addition of anti-HER2 Fab′-PEG-DSPE conjugates to the formulation (Kirpotin *et al.*, 1998). Receptor-specific targeting has also been shown to increase the amount of complexed agent reaching the nucleus. Thus, using cationic lipid complexes of antisense oligonucleotides, a significant increase in nuclear localization of fluorescein-labeled oligonucleotides was seen when anti-HER2 Fab′-PEG-DSPE conjugates were added to the composition (Meyer *et al.*, 1998).

The focus of this chapter has been on vitamin and growth factor receptors with respect to using them as targets for increasing binding and internalization of liposomes by malignant cells. However, liposomes and cationic lipid complexes can also be used to delivery therapeutic agents, such as antisense oligonucleotides or genes that code for inhibitory proteins or inhibit growth factor receptor expression by binding to nascent RNA (Wang *et al.*, 1995; Hung *et al.*, 1998; Muller *et al.*, 1998). For example, both cationic lipid–DNA complexes and adenoviral vectors have been used to deliver the E1A gene and the nontransforming simian virus 40 large-T-antigen mutant gene to tumors in both mice and humans (Yu *et al.*, 1995; Chang *et al.*, 1997; Hortobagyi *et al.*, 1998; Hung *et al.*, 1998). Both genes are thought to code for gene products that can repress HER-2/neu promoter function.

F. LIPOSOME TARGETING USING OTHER GROWTH FACTOR RECEPTORS

Liposome targeting to cells expressing NGFR, EGFR, and VEGFR has been reported. Harding *et al.* (1997) conjugated periodate-oxidized recombinant anti-EGFR immunoglobulin C225 to the liposomes coated with PEG, a portion of which was bearing terminal hydrazide groups. Increased *in vitro* uptake of anti-EGFR IgG-immunoliposomes by EGFR-expressing prostate cancer cells (DU-145) was demonstrated *in vitro*. IgG conjugation at approximately 13 IgG/molecules per liposome (100 nm) did not significantly reduce liposome circulation longevity; however, at higher IgG conjugation, the circulation half-life of the liposomes decreased threefold. Anti-EGFR IgG-immunoliposomes were highly immunogenic in rats, resulting in fast clearance upon repeated injections. No therapy studies or tumor-uptake data were presented. The targeting of both the NGF receptor and EGFR were accomplished also by using the hormone itself as a targeting ligand attached to the exterior of the liposome (Rosenberg *et al.*, 1987; Ishii *et al.*, 1987; Kikuchi *et al.*, 1996). In both studies, the growth factor-targeted liposomes were shown to be efficiently endocytosed by the target cells *in vitro*, either

fibroblasts (Ishii *et al.*, 1987) or PC12 pheochromocytoma cells (Rosen-berg *et al.*, 1987). However, no *in vivo* therapeutic approaches were attempted in these studies, leaving a considerable amount of work to be done both *in vitro* and *in vivo*. FGF has been used to target tox-ins to FGFR-overexpressing cancer cells (Beitz *et al.*, 1992; Goldman *et al.*, 1997). Adenovirus was genetically engineered to express FGF se-quences at the cell-seeking receptor sites of the capside, thus redirecting viral tropism to FGF-overexpressing cells (Gu *et al.*, 1999). Because the size of adenovirus (90 nm) is in the size range of liposomes used for tu-mor drug delivery (70–120 nm), these data suggest that FGF would be a good targeting ligand also for liposomes. Cells expressing VEGF recep-tor were also targeted using an unusual ligand, nucleic acid aptamers (Willis *et al.*, 1998) developed by the process called SELEX (Ellington and Szostak, 1990). Although promising results were obtained in in-hibiting cell proliferation in culture, the addition of the aptamers re-sulted in a dramatic decrease in circulation lifetimes when compared to nontargeted liposomes. A reduction in the number of aptamers at-tached to the liposome surface may increase circulation lifetimes for single injections. However, the potential immunogenecity of such a sys-tem may limit its usefulness if multiple injections are required. If these issues are resolved, aptamers may become a promising alternative for liposome targeting instead of more traditional peptide/protein ligands. These studies demonstrate potential of growth factor receptors in tar-geted liposome delivery, but also emphasize the importance of overall ligandoliposome design to realize the advantage of targeting in practice.

As it is the case with vitamin receptors (Section II,G), a largely unex-plored area lies in using hormone receptors for liposome targeting Pep-tide hormones and hormone-releasing factors, including plasma-stable peptide analogs and peptidomimetics, may be used as ligands to deliver liposomal drugs to endocrine tissues and hormone-responsive nonen-docrine tissues such as breast and uterus. Thus, a recent study success-fully used doxorubicin conjugate with luteinizing hormone-releasing hormone (LH-RH) to deliver the cytotoxic drug to a human prostate cancer xenograft (Koppán *et al.*, 1999). The experience of using these and other ligands reactive with hormone and growth factor receptors to-gether with the principles of rational ligandoliposome design (Table II) will help to create a new generation of liposomal drug-delivery systems.

IV. Conclusions and Future Perspectives

Due to their unique place in providing for cellular growth and dif-ferentiation, and for their ability to internalize into the cell, vitamin

and growth factor receptors undergo changes in malignant cells that make these cellular markers good recognition tags for targeted onco-logical drug delivery using liposomes. To date, despite many promising *in vitro* studies in this area, little has been accomplished *in vivo*, possi-bly because the field of liposome pharmacology has not been advanced enough to provide for a practically feasible ligandoliposome. Now, with more liposome formulations entering the oncopharmaceutical market, the development of new advanced technologies for making targeting ligands (scFv, aptamers) and their conjugates to liposomes (PEG-DSPE anchors) will allow these compounds to enter the clinic. In this chap-ter the principles of ligandoliposome targeting were outlined; we also illustrated how these principles were implemented in two successful liposome targeting systems directed to folate receptor via its natural ligand, folic acid, and to HER2/neu growth factor receptor via an anti-HER2 antibody fragment. The successful methodologies developed in these exemplary cases are easy to extrapolate onto a vast variety of ligands and lipid-based drug-delivery constructs, bringing us closer to the time when ligand-directed liposome targeting becomes a routine method in drug delivery.

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VITAMINS AND HORMONES, VOL. 60

Vitamins and Homocysteine Metabolism

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I. Introduction

Homocysteine is a thiol-containing intermediary metabolite that lies at a crossroads of three important metabolic processes: the methionine cycle, the folate cycle, and the transsulfuration pathway. In recent years, there has been increased interest in homocysteine metabolism because of the clinical observation that individuals with elevated levels of total plasma homocysteine (tHcy) are at increased risk of vascular disease and that woman with elevated tHcy levels are at increased risk of giving birth to children with neural tube defects.

Three vitamins play a key role in homocysteine metabolism: pyridoxine (B_6), cobalamin (B_{12}), and folate. Deficiency in any of these three vitamins can cause increased tHcy levels and increased risk of vascular disease and birth defects. In this chapter the current state of knowledge of homocysteine metabolism is summarized. The first section of this chapter discusses the molecular details of the role of these vitamins in the enzyme chemistry of homocysteine metabolism. The second section discusses clinical and epidemiologic data concerning the

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